Journal of Medical Entomology, 56(6), 2019, 1467–1474
doi: 10.1093/jme/tjz112
Advance Access Publication Date: 24 September 2019
Forum

Special Collection: Twenty Years of West Nile Virus in the United States

On the Fly: Interactions Between Birds, Mosquitoes, and Environment That Have Molded West Nile Virus Genomic Structure Over Two Decades

Nisha K. Duggal, [1] Kate E. Langwig, [2] Gregory D. Ebel, [3] and Aaron C. Brault[4,5,]

[1]Department of Biomedical Sciences and Pathobiology, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, [2]Department of Biological Sciences, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061, [3]Department of Microbiology, Immunology, and Pathology, Colorado State University, Fort Collins, CO 80523, [4]Division of Vector-borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO 80521, and[5]Corresponding author, e-mail: zlu5@cdc.gov

Subject Editor: William Reisen

Received 13 May 2019; Editorial decision 12 June 2019

Abstract

West Nile virus (WNV) was first identified in North America almost 20 yr ago. In that time, WNV has crossed the continent and established enzootic transmission cycles, resulting in intermittent outbreaks of human disease that have largely been linked with climatic variables and waning avian seroprevalence. During the transcontinental dissemination of WNV, the original genotype has been displaced by two principal extant genotypes which contain an envelope mutation that has been associated with enhanced vector competence by *Culex pipiens* L. (Diptera: Culicidae) and *Culex tarsalis* Coquillett vectors. Analyses of retrospective avian host competence data generated using the founding NY99 genotype strain have demonstrated a steady reduction in viremias of house sparrows over time. Reciprocally, the current genotype strains WN02 and SW03 have demonstrated an inverse correlation between house sparrow viremia magnitude and the time since isolation. These data collectively indicate that WNV has evolved for increased avian viremia while house sparrows have evolved resistance to the virus such that the relative host competence has remained constant. Intrahost analyses of WNV evolution demonstrate that selection pressures are avian species-specific and purifying selection is greater in individual birds compared with individual mosquitoes, suggesting that the avian adaptive and/or innate immune response may impose a selection pressure on WNV. Phylogenomic, experimental evolutionary systems, and models that link viral evolution with climate, host, and vector competence studies will be needed to identify the relative effect of different selective and stochastic mechanisms on viral phenotypes and the capacity of newly evolved WNV genotypes for transmission in continuously changing landscapes.

Key words: Arboviral Molecular Biology, arboviral transmission, Arbovirology, Virology, West Nile virus

Humans and other mammals are considered dead-end hosts and are not expected to contribute to the evolution of West Nile virus (WNV; Flaviviridae: Flavivirus) as the virus is maintained and amplified in an enzootic cycle between birds and mosquitoes (Reisen 2013). As such, these are the principal forces in addition to environmental factors that can significantly affect the growth of WNV in the poikilothermic vectors that have molded the genetic structure of WNV over the previous 20 yr. The subsequent sections of this forum article will specifically address the relative evolutionary forces that have influenced WNV diversification and the interplay between fitness in both of the integral but physiologically diverse hosts that WNV uses for its transmission.

West Nile Virus Evolves Under Purifying and Positive Selection

Selective pressures and stochastic effects imposed in avian and mosquito hosts define the evolutionary trajectories for WNV (Brault 2009). To maintain viral fitness in these highly divergent hosts, most WNV variants are not transmitted for many cycles because they are deleterious in at least one host. In addition, the seasonality of the viral enzootic cycle throughout much of its distribution causes large changes in the effective population size of the virus over the course of each year (Grubaugh et al. 2015, Grubaugh et al. 2016). Overwintering, which is thought to occur by vertical transmission in mosquitoes, reduces viral population size drastically through

bottlenecks, and also may serve as a potential selective source for variants that are efficiently vertically transmitted. However, the low vertical transmission rate of WNV in *Culex* mosquitoes likely results from transovular infection of the egg as it proceeds through the oviduct rather than transovarial infection of the germ line tissue (Nelms et al. 2013). As such, stochastic effects for the interaction of the virus with the egg would not as readily be subjected to selective evolutionary pressures with the exception that higher peripheral titers in female mosquitoes could facilitate transovular infection (Nelms et al. 2013). Nevertheless, specific experimental and/or field assessments of vertical infection for such selection and/or founder effects on viral populations have not been performed. A lower rate of evolution than expected relative to neutral evolution has been identified to be acting on the WNV genome over time, likely resultant from the effects of both purifying selection and seasonal reductions in population size. Experimental studies on WNV have supported this hypothesis, in which the bird-mosquito host-switching cycle was shown to lead to purifying selection acting on the viral genome over several generations (Jerzak et al. 2008, Deardorff et al. 2011). The rate of evolution of WNV in the United States has been calculated as ca. 4–5 × 10^{-4} nucleotide substitutions/site/year, although this rate has fluctuated over time (Pybus et al. 2012, Di Giallonardo et al. 2016).

Intrahost viral diversity refers to the amount of genetic diversity generated within an individual and has been measured using a variety of sequencing methods, most recently using next-generation sequencing (NGS) platforms. The amount and type of intrahost WNV diversity (e.g., measures of population richness, complexity, and divergence) generated during an infection is dependent on the host species sustaining the infection, although purifying selection is still the dominant form of selection acting on the genome. In general, the amount of viral diversity generated within individual mosquitoes is greater than the amount of viral diversity generated within the serum of individual birds (Jerzak et al. 2007, Jerzak et al. 2008). The level of WNV diversity generated is further variable within different bird and mosquito species (Grubaugh et al. 2015, Grubaugh and Ebel 2016). The stronger purifying selection pressure acting on WNV during an infection in birds compared with an infection in mosquitoes may be due to differences in the adaptive/innate immune response of birds and the innate immune response in mosquitoes to infection.

Diversifying selection also acts on the WNV genome, particularly within localized genomic regions. For example, strong positive selection has driven diversification of WNV in viral protein NS3 at amino acid 249 (Brault et al. 2007). Emergence of a proline at NS3-249 in lineage 1a WNVs has been observed on at least three independent occasions that predated the introduction of WNV into North America (NA). Reverse genetic approaches have demonstrated significant avian virulence phenotypes associated with genetic variation at the NS3-249 locus. Modification of NS3-249 from a threonine to a proline resulted in elevated viremia levels and mortality in American crows (AMCR, *Corvus brachyrhynchos* Brehm; Brault et al. 2007) that could be modulated to a lesser extent by other nonstructural protein substitutions (Dietrich et al. 2016). Reciprocally, modulation of NS3-249 from proline to a threonine, alanine, or histidine significantly reduced viremias and mortality rates in AMCRs (Langevin et al. 2014). These data indicated that increased amplification of WNV within certain avian hosts can serve as a key trigger for enhanced enzootic circulation of the viruses that subsequently result in spill-over transmission to humans. Interestingly, lineage 2 WNVs isolated during the Greek 2010–2013 epidemic/epizootic similarly exhibited a histidine to proline substitution at this same site (Papa et al. 2011).

Avian and Mosquito Immunity Drive WNV Evolution

In many birds, WNV typically produces an acute infection lasting up to a week, though viral persistence for several weeks has been shown in wild birds (Nemeth et al. 2009a, b; Wheeler et al. 2012a). Avian species vary in their initial susceptibility to infection and the severity of disease after infection, with mortality ranging from 0 to 100%. Passerines sustain the highest viremia and mortality rates, whereas gallinaceous birds manifest very low viremias and exhibit no morbidity or mortality (Komar et al. 2003, Reisen et al. 2005a). The overall effect of WNV on local passerine populations can be significant (LaDeau et al. 2007, Wheeler et al. 2009). The course of infection is likely influenced by innate immune responses, as monocytes/macrophages are thought to be early cellular targets of WNV in corvids (Dietrich et al. 2015), followed by dissemination to organs and the development of inflammatory lesions (Weingartl et al. 2004). However, the innate immune response to WNV infection has not been well characterized in birds. Adaptive immune responses, such as neutralizing antibody responses, are well-documented in many avian species against WNV and can be protective against subsequent infection for many years (Nemeth et al. 2008, 2009b). Therefore, although certain species of birds are critical for WNV enzootic maintenance due to their high viremias during infection, WNV imposes a significant cost to these bird populations due to the associated morbidity and mortality. This interaction could drive a genetic "arms race" between the virus and avian host. Indeed, evidence of this has been presented by the identification of reduced viremias in house sparrows infected with the NA founding WNV genotype over time. House sparrows [HOSP; *Passer domesticus* (L.)] from the early 2000s were found to manifest higher viremias when infected with the NY99 genotype of WNV than HOSPs collected in 2013 and inoculated with the same viral strain (Duggal et al. 2014). This finding and the reciprocal demonstration that HOSPs collected in 2012–2013 developed higher viremias with more recently isolated WNV strains, representing the WN02 and SW03 genotypes, compared with the NY99 genotype, further indicated the likely importance of selection in HOSPs for the emergence of the extant genotypes (Duggal et al. 2014). Evidence supporting the importance of HOSPs for WNV in the field include their high host competence (Komar et al. 2003, Langevin et al. 2005, Reisen et al. 2005a), the frequent identification of HOSP blood meals in competent *Culex* spp. vectors (Hamer et al. 2009, Thiemann et al. 2011, Komar et al. 2013) and the identification of suppressed transmission with elevated seroprevalence of this species (Kwan et al. 2012). However, the role of alternative avian species as a selective influence has not been assessed experimentally.

Mosquito immunity against RNA viruses is principally a response against double-stranded RNA replication intermediates and viral RNA secondary structures that are required for virus replication. This immune response is enabled by the RNA-induced silencing complex (RISC) into which cleavage products of double-stranded RNA intermediates are loaded and serve to screen the cytoplasm for the complementary sequences. As such, the activated or primed RISC is highly sequence-dependent. A number of studies have demonstrated that the sequence specificity of the mosquito innate immune response against WNV has fostered additional sequence diversity (Brackney et al. 2009, Brackney et al. 2011), reducing the efficacy of the RNAi response against any individual sequence. This genetic variation typically has manifested as first and principally third codon synonymous substitutions. The cycling of WNV between mosquito vectors that generate considerable genetic diversity and avian hosts that serve as a strong source of purifying

selection modulates the overall genetic diversity observed in WNV populations (Jerzak et al. 2005). When WNV was serially adapted to mosquitoes, viral transmission fitness was compromised due largely to the fitness costs associated with higher viral loads on the mosquito despite enhanced replicative fitness in the vector (Ciota et al. 2013).

The Mosquito Microbiome and Its Effects on Vector Competence for WNV

The role of the mosquito microbiome has emerged as an apparent source of variation that can alter vector-virus interactions, thereby modulating the competence and propensity of vectors to transmit arboviral agents such as WNV. Although considerable literature has demonstrated the role of bacterial populations, especially *Wolbachia* spp., with modulation of vector competence (Hussain et al. 2013), growing evidence has demonstrated that the mosquito microvirome can specifically modulate transmissibility of arboviruses such as WNV. Insect-specific flaviviruses such as Culex flavivirus (CxFV; *Flaviviridae: Flavivirus*), that are phylogenetically ancestral to the mosquito-borne flavivirus lineage and do not replicate in vertebrates, only modestly affect infections and/or transmission with other flaviviruses such as WNV. In fact, a positive association between individual female *Culex pipiens* L. (Diptera: Culicidae) infected with WNV and positivity for CxFV in the Chicago area may indicate an enhanced phenotype either through innate immune modulation by heterologous viruses or enhanced vertical infection rates (Newman et al. 2011). The lack of a superinfection barrier readily observed for CxFV and WNV likely is based on the low genetic identity between these viruses. In contrast, Nhumirim virus (NHUV; *Flaviviridae: Flavivirus*), an insect-specific flavivirus that seems to have recently lost the ability to replicate in vertebrates (Kenney et al. 2014), potently inhibits WNV both in vitro and in vivo, reducing the transmissibility of WNV in *Culex quinquefasciatus* Say (Goenaga et al. 2015). A similar finding has been demonstrated in NHUV-infected *Aedes aegypti* (L.) orally exposed to Zika virus (*Flaviviridae*: *Flavivirus*; Romo et al. 2018). The selective pressures associated with coinfection of heterologous flaviviruses could play a significant role in modulating the genetic diversity and subsequent phenotypes of WNV.

In cells coinfected with a replicon expressing WNV RNA, a specific mutation (V9M) within the 2K protein was associated with an increased capacity to replicate in the presence of competing WNV RNA populations (Zou et al. 2009a,b). Interestingly, this resistance to superinfection exclusion does not extend to coinfections with a heterologous flavivirus (Goenaga et al. 2015). These data indicated that, although intracellular competition of WNV with other viruses present within the mosquito microvirome could serve as a selective pressure for WNV and other viruses of human health importance, this likely results from complex interactions dictated by specific competition influences and will require considerable study to identify its potential effects on WNV genetic structure and subsequent phenotypes. This complexity has been further demonstrated by the finding that WNV exposure/infection of *Cx. pipiens* can alter bacterial microbiota diversity, thus having additional effects on immune pathway activation and potential altered susceptibility to alternative pathogens of human health importance (Zink et al. 2015).

Potential Effects of Overwintering and Viral Persistence on the WNV Adaptive Landscape

The source of WNV in a given geographic environment in NA has been a point of controversy; however, the genetic similarity between WNV sequences derived from the fall and the subsequent spring transmission seasons (Duggal et al. 2015) has indicated the likely maintenance of viruses in the same geographic location either by overwintering or by reintroduction form a similar locale. Evidence for several persistence mechanisms has been identified. In warmer climates such as in southern California, low-level year-round transmission can be enabled with warmer temperatures. Alternatively, vertically infected female mosquitoes that did not take a bloodmeal in the fall can overwinter as nulliparous diapausing females and can transmit virus following their initial bloodmeal in the spring, thereby reestablishing transmission (Reisen et al. 2006a). Bird-to-bird transmission initially was proposed as an overwintering mechanism in cooler areas not conducive for continual mosquito-bird transmission over winter months following the isolation of WNV from a dead hawk in the winter in New York in 2000 and 2004–2005 (Garmendia et al. 2000, Dawson et al. 2007). During the winter months, in which competent vectors are absent or in insufficient numbers to support purely vector-borne transmission, transmission could be perpetuated by persistent infection in avian hosts with periodic recrudescence during time periods more amenable for vector transmission. A number of studies have sought to assess the potential for persistent infection in different avian species (Nemeth et al. 2009a, Wheeler et al. 2012b). Although viral RNA could be consistently identified in these birds for up to 36 wk, infectious virus was seldomly identified beyond 30 d postinoculation. Persistence in multiple tissues including neural tissue has also been documented in experimentally infected mice with recrudescence following cyclophosphamide immune suppression (Appler et al. 2010). Other potential mechanisms that could allow for overwintering transmission include the periodic emergence of infected females from overwintering hibernacula during warmer periods of the winter (Nasci et al. 2001, Andreadis et al. 2010, Ciota et al. 2011) as well as ectoparasite transmission between birds (Oesterle et al. 2010). Although phylogeographic studies with WNV strains isolated in the fall and the subsequent spring have shown a high degree of genetic conservation (Duggal et al. 2015), suggestive of maintenance of previously circulating strains in a geographic area, specific genetic signatures have not been identified indicating that this persistence was perpetuated by persistent infection in specific avian populations. Long-term viral replication within individual birds has implications for WNV evolution, as the virus may adapt to the host adaptive immune responses during a chronic infection.

Avian consumption of infected dead bird carcasses (Komar et al. 2003) has also been proposed as a possible means for avian infection during the winter months. Direct oral exposure of AMCRs to WNV infected HOSPs has provided evidence for potential bird-to-bird transmission (Komar et al. 2003).Cage mate transmission has been observed between AMCRs for which high-titered infectious virus was isolated from cloacal swabs (Komar et al. 2002), feces, and waterers of cohoused birds (Kipp et al. 2006). The potential for fecal-oral transmission has been emphasized for its relevance to possible implications for bird-to-bird transmission at communal roosting sites (Dawson et al. 2007). In one study in which crow roosting sites were sampled over winter months, dead crows positive for WNV were identified repeatedly during time periods in which mosquito abundance and infection rates were absent or exceedingly low (Hinton et al. 2015). The high frequency of moribund or dead raptors with WNV diagnosis could be a manifestation of a trophic amplification through either the ingestion of infected birds or consumption of carrion as well as enhanced surveillance through the specific employment of raptor rehabilitation centers (Nemeth et al. 2007, Smith et al. 2018). Communal roosting behavior of some avian species such as American robins and ardeids has been shown

to not be associated with higher transmission (Reisen et al. 2005b, Benson et al. 2012) and others have been associated with diminished WNV circulation (Komar et al. 2015). Reduced circulation of WNV in proximity to great-tailed grackle (*Quiscalus mexicanus* (Gmelin) roosts in Maricopa county, Arizona was proposed to have resulted from zooprophylaxis from the accumulation of avian immunity (Komar et al. 2015). Nevertheless, other studies have shown roosting behavior to facilitate transmission in different areas such as roosting sites over land (Reisen et al. 2009) or in different geographic areas or with alternative avian species (Reisen et al. 2006b, Diuk-Wasser et al. 2010). Mosquito-to-mosquito or bird-to-bird transmission of WNV has implications for WNV evolution, as the WNV adaptive landscape would no longer be constrained by a dual-host cycle.

Impact of WNV Genetic Variants on Viral Transmission Potential

During the spread of WNV across the United States, one nonsynonymous mutation has become fixed in the viral population (E-V159A) and an additional nonsynonymous substitution (NS4A-A85T) has reached a high frequency in western NA. The valine-to-alanine mutation in the envelope protein at E-159, which is referred to as the WN02 genotype, was first detected in 2002 in Texas (Beasley et al. 2003) and has been present in every NA isolate sequenced since 2004 (Davis et al. 2005). Incorporation of an additional alanine-to-threonine mutation in the NS4A protein at amino acid 85 (NS4A-85) in WN02 genotype viruses has occurred on several occasions. This variant is referred to as SW03 and was first detected in 2003 in Texas and has been found in isolates from across the United States (Fig. 1; Mann et al. 2013, Duggal et al. 2015, Di Giallonardo et al. 2016); however, sympatric circulation of WN02 viruses with and without the NS4A substitution has been shown to occur in NA (Duggal et al. 2013, Duggal et al. 2014). The original viral genotype that lacks these two mutations is referred to as the NY99 genotype. Additional nonsynonymous mutations have been identified in many WNV isolates; however, they have been restricted geographically or temporally. Phylogeographic studies have calculated the spread of WNV across the United States as extremely heterogeneous and diffusive during the initial spread, with diffusion rates >1,000 km^2/d (Pybus et al. 2012). Subsequent phylogeographic studies showed a significantly increased dispersal rate of SW03 genotype viruses relative to WN02 genotype viruses in California, suggesting an increased fitness of the SW03 genotype (Fig. 1; Duggal et al. 2015); however, to date, no specific phenotypic advantage has been observed for viruses containing the NS4A SW03 mutation.

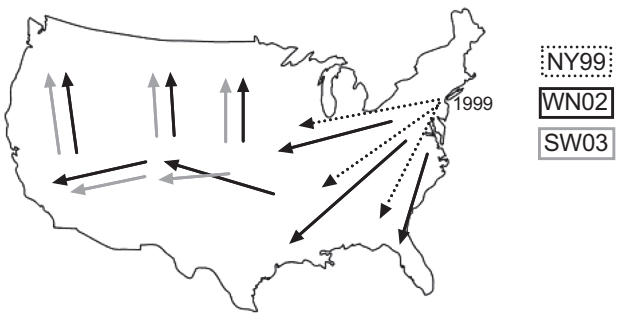

Fig. 1. Depiction of WNV genotypes spreading across the United States, beginning with the first report of WNV (NY99 genotype, dashed black arrows) in New York in 1999. The NY99 genotype was displaced by the WN02 genotype (black solid arrows), and the WN02 and SW03 genotypes (gray solid arrows) are currently cocirculating. Initial WNV strains isolated in Mexico in 2003 were from the East coast genotype (Deardorff et al. 2006) with subsequent isolates representative of the WN02 genotype (Blitvich et al. 2004, Elizondo-Quiroga et al. 2005).

Experimental vector studies have compared the competence of NY99 genotype viruses versus WN02 genotype viruses for infection, dissemination, and transmission by several *Culex* species mosquitoes including *Cx. quinquefasciatus*, *Cx. pipiens*, and *Culex tarsalis* Coquillett (Diptera: Culicidae). These vectors have been shown to be the predominant WNV vectors in southern, northern, and western United States, respectively (Reisen 2013). A shortened extrinsic incubation period (EIP) of WN02 genotype viruses compared to with genotype viruses has been shown in *Cx. pipiens* (Ebel et al. 2004) and this shortened incubation time was further accentuated by increased temperature (Kilpatrick et al. 2008). Similarly, a higher proportion of *Cx. tarsalis* were infected perorally with WN02 viruses compared with NY99 and transmission occurred earlier and more efficiently with the emergent genotype virus in this species (Moudy et al. 2007). This phenotype was not observed with WN02 isolates in *Cx. quinquefasciatus* mosquitoes (Richards et al. 2014), indicating the likely initial selection of this mutation for enhanced competence in *Cx. pipiens*. The subsequent radiation of the WN02 and SW03 viruses into areas of the southern United States where *Cx. quinquefasciatus* serve as the primary vectors occurred rapidly but likely not as a result of enhanced vector competence in this species. Interestingly, when vector competence assessments were performed between the NY99 genotype and subsequent Californian WNV isolates between 2003 and 2011 in Californian *Cx. tarsalis,* no difference in EIPs were observed (Danforth et al. 2015), indicating that this temperature-mediated phenomenon might not be universal. Moreover, it may be that an initial, short-term, adaptive advantage of WN02 over NY99 resulted in a cascade of transmission that resulted in the current distribution of WNV genotypes.

Experimental inoculations in wild birds have assessed the relative host competence of WNV isolates collected over time. In HOSPs, WN02 and SW03 genotype isolates reached higher peak viremias than NY99 genotype viruses (Duggal et al. 2014). In in vivo competition experiments in house finches [HOFI; *Haemorhous mexicanus* (Müller)], WNV isolates collected more recently had higher or neutral fitness relative to older WNV isolates (Worwa et al. 2018, 2019). Although WNV isolates have become more viremia-inducing in wild birds, when the viral genotype was held constant, contemporary HOSPs and HOFIs sustained lower viremias after infection compared with birds from a decade ago (Duggal et al. 2014, Worwa et al. 2019). This suggested ongoing coevolution of the avian host for increased resistance to WNV, with the net consequence of no change in host competence for contemporary birds for contemporary viruses, and was consistent with an ongoing genetic arms-race between WNV and birds. Future analyses could examine the dual influence of evolutionary changes in hosts and viruses in a dynamic modeling framework to provide additional insight into the net consequences of evolutionary changes in hosts and viruses.

What is Driving the Evolution of WNV?

Since its emergence, WNV has become the most important arbovirus in the continental United States, with unpredictable outbreaks occurring during spillover of enzootic transmission to humans. Identifying the factors that affect WNV evolution during the enzootic cycle may help us to explain the causes of WNV outbreaks in humans. Currently, our understanding of WNV evolution suggests viral diversity increases in mosquitoes and decreases in birds, with factors affecting viral evolution including innate immunity,

coinfection, and overwintering in mosquitoes and innate/adaptive immunity and viral persistence in birds (Fig. 2). To further understand WNV evolution, there are large scientific knowledge gaps that could be addressed by combining data from experimental evolutionary systems with ecological assessments.

The avian immune response to WNV infection, which determines the susceptibility to morbidity/mortality of birds, and therefore affects how selection shapes WNV, has not been well characterized. To study the avian immune response, tools for passerines are needed, including more full-genome sequences of birds. This will allow for the development of real-time or flow cytometry assays to measure the innate immune response of multiple avian species to WNV infection by different viral genotypes. Chickens, for which most avian immune reagents have been generated, are not an ideal model for studying the immune response to WNV infection, as only very young chicks are susceptible to infection, and the pathogenesis of WNV observed during infection in passerines is not reproduced in chickens.

Parallels can be drawn between the evolutionary pressures and resultant WNV genetic population structures that have emerged in the past 20 yr since WNV was first observed in NA and endemic NA arboviruses with a much longer evolutionary history with resident avian and mosquito fauna. The alphavirus, eastern equine encephalitis virus (EEEV), also transmitted among passeriform birds, has been shown to have a much lower nucleotide substitution rate in NA compared with South American EEEV variants (Madariaga virus) transmitted among rodents (Brault et al. 1999, Weaver et al. 1991, Arrigo et al. 2010). This lower evolutionary rate, presumably resulting from the efficient dispersal of NA EEEV genetic variants by birds, has diminished the kinds of population partitioning that would be expected to result in genetic drift evident in geographically isolated EEEV populations transmitted by rodents. West Nile viruses have shown similar substitution rates as those of NA EEEV (Añez et al. 2013) likely the result of similar avian dispersal mechanisms (Swetnam et al. 2018). A NA endemic flavivirus that utilizes similar avian and mosquito vectors as WNV, Saint Louis encephalitis virus (SLEV), has demonstrated very high oral infectivity as a likely means of efficient transmission, despite the development of relatively low avian titers compared with WNV. The nonstructural genes of SLEV have been implicated with this high-vector infection phenotype (Maharaj et al. 2014). Interestingly, in vitro studies have demonstrated that SLEV comparatively maintains a much more genetically homogeneous population structure compared with WNV (Ciota et al. 2007). This enhanced genetic stability could signify a reduced need for maintaining sequence diversity in the mosquito vector for avoidance of the RNAi response which has been evident with WNV (Brackney et al. 2009). Future studies should address whether nonstructural modifications within WNV could alter the location or kinetics of RNA replication within the mosquito vector such that less sequence heterogeneity would be generated in WNV-infected mosquitoes. Despite significant progress in addressing the evolutionary impacts of different host types on WNV evolutionary dynamics, significant holes remain in our understanding of how distinct transmission ecologies affect virus evolution. Assessing this is increasingly important because new arboviruses continue to emerge as human health threats and WNV represents a unique system where both the mosquito and vertebrate portions of the transmission cycle can be modeled in the laboratory. Additional opportunities exist in the WNV system for addressing how host susceptibility influences the strength of selection during replication.

Mathematical models integrating the evolutionary responses of hosts, vectors, and viral strains could provide novel insights into the cumulative effects of evolutionary change on WNV transmission. Incorporating these effects into spatial models addressing variation in geography and climate (Paull et al. 2017) may reveal dual effects of both genetics and the environment on WNV dynamics. For example, climatic conditions that favor vectors may increase selection for host resistance, creating geographic mosaics (Thompson 2005) of coevolving hosts and pathogen strains. As described in this forum, laboratory experiments and field studies have revealed significant evolutionary changes during the course of WNV invasion, and models integrating variation in host resistance (Langwig et al. 2017) with pathogen evolution (Fleming-Davies et al. 2018) may help explain changes in WNV dynamics across space and time.

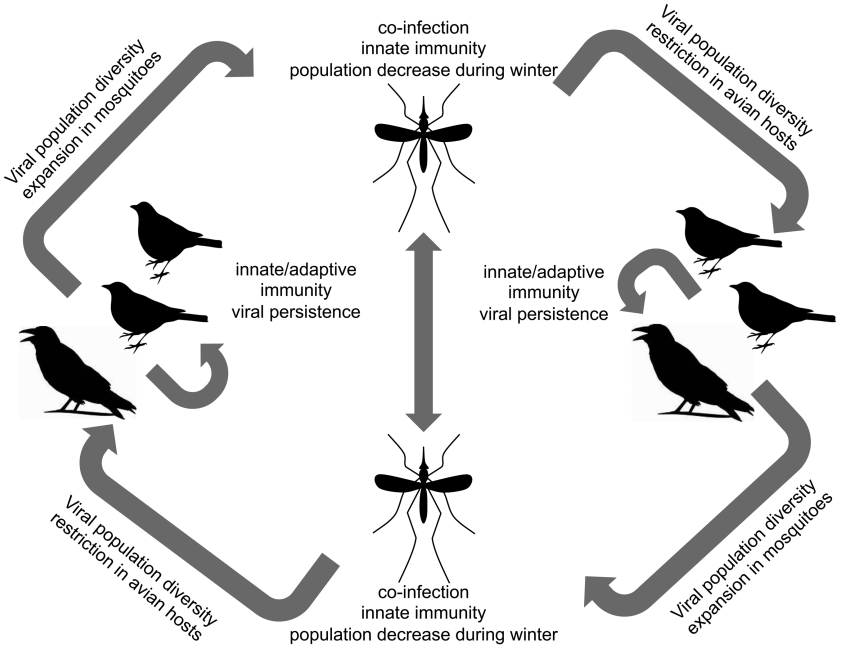

Fig. 2. Transmission of WNV and the selective criteria that have molded the genetic structure of the virus over time.

Acknowledgments

Funding was provided by the National Institutes of Health R01GM113233 and AI067380.

References Cited

Andreadis, T. G., P. M. Armstrong, and W. I. Bajwa. 2010. Studies on hibernating populations of Culex pipiens from a West Nile virus endemic focus in New York City: parity rates and isolation of West Nile virus. J. Am. Mosq. Control Assoc. 26: 257–264.

Añez, G., A. Grinev, C. Chancey, C. Ball, N. Akolkar, K. J. Land, V. Winkelman, S. L. Stramer, L. D. Kramer, and M. Rios. 2013. Evolutionary dynamics of West Nile virus in the United States, 1999-2011: phylogeny, selection pressure and evolutionary time-scale analysis. Plos Negl. Trop. Dis. 7: e2245.

Appler, K. K., A. N. Brown, B. S. Stewart, M. J. Behr, V. L. Demarest, S. J. Wong, and K. A. Bernard. 2010. Persistence of West Nile virus in the central nervous system and periphery of mice. PLoS One 5: e10649.

Arrigo, N. C., A. P. Adams, and S. C. Weaver. 2010. Evolutionary patterns of eastern equine encephalitis virus in North versus South America suggest ecological differences and taxonomic revision. J. Virol. 84: 1014–1025.

Beasley, D. W., C. T. Davis, H. Guzman, D. L. Vanlandingham, A. P. Travassos da Rosa, R. E. Parsons, S. Higgs, R. B. Tesh, and A. D. Barrett. 2003. Limited evolution of West Nile virus has occurred during its southwesterly spread in the United States. Virology. 309: 190–195.

Benson, T. J., M. P. Ward, R. L. Lampman, A. Raim, and P. J. Weatherhead. 2012. Implications of spatial patterns of roosting and movements of American robins for West Nile virus transmission. Vector Borne Zoonotic Dis. 12: 877–885.

Blitvich, B. J., I. Fernández-Salas, J. F. Contreras-Cordero, M. A. Loroño-Pino, N. L. Marlenee, F. J. Díaz, J. I. González-Rojas, N. Obregón-Martínez, J. A. Chiu-García, W. C. Black, 4th, et al. 2004. Phylogenetic analysis of West Nile virus, Nuevo Leon State, Mexico. Emerg. Infect. Dis. 10: 1314–1317.

Brackney, D. E., J. E. Beane, and G. D. Ebel. 2009. RNAi targeting of West Nile virus in mosquito midguts promotes virus diversification. PLoS Pathog. 5: e1000502.

Brackney, D. E., K. N. Pesko, I. K. Brown, E. R. Deardorff, J. Kawatachi, and G. D. Ebel. 2011. West Nile virus genetic diversity is maintained during transmission by *Culex pipiens quinquefasciatus* mosquitoes. PLoS One 6: e24466.

Brault, A. C. 2009. Changing patterns of West Nile virus transmission: altered vector competence and host susceptibility. Vet. Res. 40: 43.

Brault, A. C., A. M. Powers, C. L. Chavez, R. N. Lopez, M. F. Cachón, L. F. Gutierrez, W. Kang, R. B. Tesh, R. E. Shope, and S. C. Weaver. 1999. Genetic and antigenic diversity among eastern equine encephalitis viruses from North, Central, and South America. Am. J. Trop. Med. Hyg. 61: 579–586.

Brault, A. C., C. Y. Huang, S. A. Langevin, R. M. Kinney, R. A. Bowen, W. N. Ramey, N. A. Panella, E. C. Holmes, A. M. Powers, and B. R. Miller. 2007. A single positively selected West Nile viral mutation confers increased virogenesis in American crows. Nat. Genet. 39: 1162–1166.

Ciota, A. T., A. O. Lovelace, S. A. Jones, A. Payne, and L. D. Kramer. 2007. Adaptation of two flaviviruses results in differences in genetic heterogeneity and virus adaptability. J. Gen. Virol. 88: 2398–2406.

Ciota, A. T., C. L. Drummond, J. Drobnack, M. A. Ruby, L. D. Kramer, and G. D. Ebel. 2011. Emergence of *Culex pipiens* from overwintering hibernacula. J. Am. Mosq. Control Assoc. 27: 21–29.

Ciota, A. T., D. J. Ehrbar, A. C. Matacchiero, G. A. Van Slyke, and L. D. Kramer. 2013. The evolution of virulence of West Nile virus in a mosquito vector: implications for arbovirus adaptation and evolution. BMC Evol. Biol. 13: 71.

Danforth, M. E., W. K. Reisen, and C. M. Barker. 2015. Extrinsic incubation rate is not accelerated in recent California Strains of West Nile virus in *Culex tarsalis* (Diptera: Culicidae). J. Med. Entomol. 52: 1083–1089.

Davis, C. T., G. D. Ebel, R. S. Lanciotti, A. C. Brault, H. Guzman, M. Siirin, A. Lambert, R. E. Parsons, D. W. Beasley, R. J. Novak, et al. 2005. Phylogenetic analysis of North American West Nile virus isolates, 2001-2004: evidence for the emergence of a dominant genotype. Virology. 342: 252–265.

Dawson, J. R., W. B. Stone, G. D. Ebel, D. S. Young, D. S. Galinski, J. P. Pensabene, M. A. Franke, M. Eidson, and L. D. Kramer. 2007. Crow deaths caused by West Nile virus during winter. Emerg. Infect. Dis. 13: 1912–1914.

Deardorff, E., J. Estrada-Franco, A. C. Brault, R. Navarro-Lopez, A. Campomanes-Cortes, P. Paz-Ramirez, M. Solis-Hernandez, W. N. Ramey, C. T. Davis, D. W. Beasley, et al. 2006. Introductions of West Nile virus strains to Mexico. Emerg. Infect. Dis. 12: 314–318.

Deardorff, E. R., K. A. Fitzpatrick, G. V. Jerzak, P. Y. Shi, L. D. Kramer, and G. D. Ebel. 2011. West Nile virus experimental evolution *in vivo* and the trade-off hypothesis. PLoS Pathog. 7: e1002335.

Dietrich, E. A., R. A. Bowen, and A. C. Brault. 2015. An ex vivo avian leukocyte culture model for West Nile virus infection. J. Virol. Methods 218: 19–22.

Dietrich, E. A., S. A. Langevin, C. Y. Huang, P. D. Maharaj, M. J. Delorey, R. A. Bowen, R. M. Kinney, and A. C. Brault. 2016. West Nile Virus temperature sensitivity and avian virulence are modulated by NS1-2B polymorphisms. Plos Negl. Trop. Dis. 10: e0004938.

Di Giallonardo, F., J. L. Geoghegan, D. E. Docherty, R. G. McLean, M. C. Zody, J. Qu, X. Yang, B. W. Birren, C. M. Malboeuf, R. M. Newman, et al. 2016. Fluid spatial dynamics of West Nile Virus in the United States: rapid spread in a permissive host environment. J. Virol. 90: 862–872.

Diuk-Wasser, M. A., G. Molaei, J. E. Simpson, C. M. Folsom-O'Keefe, P. M. Armstrong, and T. G. Andreadis. 2010. Avian communal roosts as amplification foci for West Nile virus in urban areas in northeastern United States. Am. J. Trop. Med. Hyg. 82: 337–343.

Duggal, N. K., M. D'Anton, J. Xiang, R. Seiferth, J. Day, R. Nasci, and A. C. Brault. 2013. Sequence analyses of 2012 West Nile virus isolates from Texas fail to associate viral genetic factors with outbreak magnitude. Am. J. Trop. Med. Hyg. 89: 205–210.

Duggal, N. K., A. Bosco-Lauth, R. A. Bowen, S. S. Wheeler, W. K. Reisen, T. A. Felix, B. R. Mann, H. Romo, D. M. Swetnam, A. D. Barrett, et al. 2014. Evidence for co-evolution of West Nile Virus and house sparrows in North America. Plos Negl. Trop. Dis. 8: e3262.

Duggal, N. K., W. K. Reisen, Y. Fang, R. M. Newman, X. Yang, G. D. Ebel, and A. C. Brault. 2015. Genotype-specific variation in West Nile virus dispersal in California. Virology. 485: 79–85.

Ebel, G. D., J. Carricaburu, D. Young, K. A. Bernard, and L. D. Kramer. 2004. Genetic and phenotypic variation of West Nile virus in New York, 2000-2003. Am. J. Trop. Med. Hyg. 71: 493–500.

Elizondo-Quiroga, D., C. T. Davis, I. Fernandez-Salas, R. Escobar-Lopez, D. Velasco Olmos, L. C. Soto Gastalum, M. Aviles Acosta, A. Elizondo-Quiroga, J. I. Gonzalez-Rojas, J. F. Contreras Cordero, et al. 2005. West Nile Virus isolation in human and mosquitoes, Mexico. Emerg. Infect. Dis. 11: 1449–1452.

Fleming-Davies, A. E., P. D. Williams, A. A. Dhondt, A. P. Dobson, W. M. Hochachka, A. E. Leon, D. H. Ley, E. E. Osnas, and D. M. Hawley. 2018. Incomplete host immunity favors the evolution of virulence in an emergent pathogen. Science. 359: 1030–1033.

Garmendia, A. E., H. J. Van Kruiningen, R. A. French, J. F. Anderson, T. G. Andreadis, A. Kumar, and A. B. West. 2000. Recovery and identification of West Nile virus from a hawk in winter. J. Clin. Microbiol. 38: 3110–3111.

Goenaga, S., J. L. Kenney, N. K. Duggal, M. Delorey, G. D. Ebel, B. Zhang, S. C. Levis, D. A. Enria, and A. C. Brault. 2015. Potential for co-infection of a mosquito-specific flavivirus, nhumirim virus, to Block West Nile virus transmission in mosquitoes. Viruses. 7: 5801–5812.

Grubaugh, N. D., and G. D. Ebel. 2016. Dynamics of West Nile virus evolution in mosquito vectors. Curr. Opin. Virol. 21: 132–138.

Grubaugh, N. D., D. R. Smith, D. E. Brackney, A. M. Bosco-Lauth, J. R. Fauver, C. L. Campbell, T. A. Felix, H. Romo, N. K. Duggal, E. A. Dietrich, et al. 2015. Experimental evolution of an RNA virus in wild birds: evidence for host-dependent impacts on population structure and competitive fitness. PLoS Pathog. 11: e1004874.

Grubaugh, N. D., J. Weger-Lucarelli, R. A. Murrieta, J. R. Fauver, S. M. Garcia-Luna, A. N. Prasad, W. C. Black, 4th, and G. D. Ebel. 2016. Genetic drift

during systemic arbovirus infection of mosquito vectors leads to decreased relative fitness during host switching. Cell Host Microbe 19: 481–492.

Hamer, G. L., U. D. Kitron, T. L. Goldberg, J. D. Brawn, S. R. Loss, M. O. Ruiz, D. B. Hayes, and E. D. Walker. 2009. Host selection by *Culex pipiens* mosquitoes and West Nile virus amplification. Am. J. Trop. Med. Hyg. 80: 268–278.

Hinton, M. G., W. K. Reisen, S. S. Wheeler, and A. K. Townsend. 2015. West Nile Virus activity in a winter roost of American Crows (*Corvus brachyrhynchos*): is bird-to-bird transmission important in persistence and amplification? J. Med. Entomol. 52: 683–692.

Hussain, M., G. Lu, S. Torres, J. H. Edmonds, B. H. Kay, A. A. Khromykh, and S. Asgari. 2013. Effect of Wolbachia on replication of West Nile virus in a mosquito cell line and adult mosquitoes. J. Virol. 87: 851–858.

Jerzak, G., K. A. Bernard, L. D. Kramer, and G. D. Ebel. 2005. Genetic variation in West Nile virus from naturally infected mosquitoes and birds suggests quasispecies structure and strong purifying selection. J. Gen. Virol. 86: 2175–2183.

Jerzak, G. V., K. Bernard, L. D. Kramer, P. Y. Shi, and G. D. Ebel. 2007. The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice. Virology. 360: 469–476.

Jerzak, G. V., I. Brown, P. Y. Shi, L. D. Kramer, and G. D. Ebel. 2008. Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching. Virology. 374: 256–260.

Kenney, J. L., O. D. Solberg, S. A. Langevin, and A. C. Brault. 2014. Characterization of a novel insect-specific flavivirus from Brazil: potential for inhibition of infection of arthropod cells with medically important flaviviruses. J. Gen. Virol. 95: 2796–2808.

Kilpatrick, A. M., M. A. Meola, R. M. Moudy, and L. D. Kramer. 2008. Temperature, viral genetics, and the transmission of West Nile virus by *Culex pipiens* mosquitoes. PLoS Pathog. 4: e1000092.

Kipp, A. M., J. A. Lehman, R. A. Bowen, P. E. Fox, M. R. Stephens, K. Klenk, N. Komar, and M. L. Bunning. 2006. West Nile virus quantification in feces of experimentally infected American and fish crows. Am. J. Trop. Med. Hyg. 75: 688–690.

Komar, N., R. Lanciotti, R. Bowen, S. Langevin, and M. Bunning. 2002. Detection of West Nile virus in oral and cloacal swabs collected from bird carcasses. Emerg. Infect. Dis. 8: 741–742.

Komar, N., S. Langevin, S. Hinten, N. Nemeth, E. Edwards, D. Hettler, B. Davis, R. Bowen, and M. Bunning. 2003. Experimental infection of North American birds with the New York 1999 strain of West Nile virus. Emerg. Infect. Dis. 9: 311–322.

Komar, N., N. A. Panella, G. R. Young, A. C. Brault, and C. E. Levy. 2013. Avian hosts of West Nile virus in Arizona. Am. J. Trop. Med. Hyg. 89: 474–481.

Komar, N., J. M. Colborn, K. Horiuchi, M. Delorey, B. Biggerstaff, D. Damian, K. Smith, and J. Townsend. 2015. Reduced West Nile Virus transmission around communal roosts of great-tailed grackle (*Quiscalus mexicanus*). Ecohealth. 12: 144–151.

Kwan, J. L., S. Kluh, and W. K. Reisen. 2012. Antecedent avian immunity limits tangential transmission of West Nile virus to humans. PLoS One 7: e34127.

LaDeau, S. L., A. M. Kilpatrick, and P. P. Marra. 2007. West Nile virus emergence and large-scale declines of North American bird populations. Nature. 447: 710–713.

Langevin, S. A., A. C. Brault, N. A. Panella, R. A. Bowen, and N. Komar. 2005. Variation in virulence of West Nile virus strains for house sparrows (*Passer domesticus*). Am. J. Trop. Med. Hyg. 72: 99–102.

Langevin, S. A., R. A. Bowen, W. K. Reisen, C. C. Andrade, W. N. Ramey, P. D. Maharaj, M. Anishchenko, J. L. Kenney, N. K. Duggal, H. Romo, et al. 2014. Host competence and helicase activity differences exhibited by West Nile viral variants expressing NS3-249 amino acid polymorphisms. PLoS One 9: e100802.

Langwig, K. E., J. R. Hoyt, K. L. Parise, W. F. Frick, J. T. Foster, and A. M. Kilpatrick. 2017. Resistance in persisting bat populations after white-nose syndrome invasion. Philos Trans R Soc Lond B Biol Sci 372..

Maharaj, P. D., B. G. Bolling, M. Anishchenko, W. K. Reisen, and A. C. Brault. 2014. Genetic determinants of differential oral infection phenotypes of West Nile and St. Louis encephalitis viruses in *Culex* spp. mosquitoes. Am. J. Trop. Med. Hyg. 91: 1066–1072.

Mann, B. R., A. R. McMullen, D. M. Swetnam, V. Salvato, M. Reyna, H. Guzman, R. Bueno, Jr, J. A. Dennett, R. B. Tesh, and A. D. Barrett. 2013. Continued evolution of West Nile virus, Houston, Texas, USA, 2002-2012. Emerg. Infect. Dis. 19: 1418–1427.

Moudy, R. M., M. A. Meola, L. L. Morin, G. D. Ebel, and L. D. Kramer. 2007. A newly emergent genotype of West Nile virus is transmitted earlier and more efficiently by *Culex* mosquitoes. Am. J. Trop. Med. Hyg. 77: 365–370.

Nasci, R. S., H. M. Savage, D. J. White, J. R. Miller, B. C. Cropp, M. S. Godsey, A. J. Kerst, P. Bennett, K. Gottfried, and R. S. Lanciotti. 2001. West Nile virus in overwintering *Culex* mosquitoes, New York City, 2000. Emerg. Infect. Dis. 7: 742–744.

Nelms, B. M., E. Fechter-Leggett, B. D. Carroll, P. Macedo, S. Kluh, and W. K. Reisen. 2013. Experimental and natural vertical transmission of West Nile virus by California *Culex* (Diptera: Culicidae) mosquitoes. J. Med. Entomol. 50: 371–378.

Nemeth, N., G. Kratz, E. Edwards, J. Scherpelz, R. Bowen, and N. Komar. 2007. Surveillance for West Nile virus in clinic-admitted raptors, Colorado. Emerg. Infect. Dis. 13: 305–307.

Nemeth, N. M., G. E. Kratz, R. Bates, J. A. Scherpelz, R. A. Bowen, and N. Komar. 2008. Naturally induced humoral immunity to West Nile virus infection in raptors. Ecohealth. 5: 298–304.

Nemeth, N., G. Young, C. Ndaluka, H. Bielefeldt-Ohmann, N. Komar, and R. Bowen. 2009a. Persistent West Nile virus infection in the house sparrow (*Passer domesticus*). Arch. Virol. 154: 783–789.

Nemeth, N. M., P. T. Oesterle, and R. A. Bowen. 2009b. Humoral immunity to West Nile virus is long-lasting and protective in the house sparrow (*Passer domesticus*). Am. J. Trop. Med. Hyg. 80: 864–869.

Newman, C. M., F. Cerutti, T. K. Anderson, G. L. Hamer, E. D. Walker, U. D. Kitron, M. O. Ruiz, J. D. Brawn, and T. L. Goldberg. 2011. Culex flavivirus and West Nile virus mosquito coinfection and positive ecological association in Chicago, United States. Vector Borne Zoonotic Dis. 11: 1099–1105.

Oesterle, P., N. Nemeth, G. Young, N. Mooers, S. Elmore, R. Bowen, P. Doherty, J. Hall, R. McLean, and L. Clark. 2010. Cliff swallows, swallow bugs, and West Nile virus: an unlikely transmission mechanism. Vector Borne Zoonotic Dis. 10: 507–513.

Papa, A., T. Bakonyi, K. Xanthopoulou, A. Vázquez, A. Tenorio, and N. Nowotny. 2011. Genetic characterization of West Nile virus lineage 2, Greece, 2010. Emerg. Infect. Dis. 17: 920–922.

Paull, S. H., D. E. Horton, M. Ashfaq, D. Rastogi, L. D. Kramer, N. S. Diffenbaugh, and A. M. Kilpatrick. 2017. Drought and immunity determine the intensity of West Nile virus epidemics and climate change impacts. Proc. Biol. Sci. 284.

Pybus, O. G., M. A. Suchard, P. Lemey, F. J. Bernardin, A. Rambaut, F. W. Crawford, R. R. Gray, N. Arinaminpathy, S. L. Stramer, M. P. Busch, et al. 2012. Unifying the spatial epidemiology and molecular evolution of emerging epidemics. Proc. Natl. Acad. Sci. U S A. 109: 15066–15071.

Reisen, W. K. 2013. Ecology of West Nile virus in North America. Viruses. 5: 2079–2105.

Reisen, W. K., Y. Fang, and V. M. Martinez. 2005a. Avian host and mosquito (Diptera: Culicidae) vector competence determine the efficiency of West Nile and St. Louis encephalitis virus transmission. J. Med. Entomol. 42: 367–375.

Reisen, W. K., S. S. Wheeler, S. Yamamoto, Y. Fang, and S. Garcia. 2005b. Nesting Ardeid colonies are not a focus of elevated West Nile virus activity in southern California. Vector Borne Zoonotic Dis. 5: 258–266.

Reisen, W. K., Y. Fang, H. D. Lothrop, V. M. Martinez, J. Wilson, P. Oconnor, R. Carney, B. Cahoon-Young, M. Shafii, and A. C. Brault. 2006a. Overwintering of West Nile virus in Southern California. J. Med. Entomol. 43: 344–355.

Reisen, W. K., C. M. Barker, R. Carney, H. D. Lothrop, S. S. Wheeler, J. L. Wilson, M. B. Madon, R. Takahashi, B. Carroll, S. Garcia, et al. 2006b. Role of corvids in epidemiology of west Nile virus in southern California. J. Med. Entomol. 43: 356–367.

Reisen, W. K., S. Wheeler, M. V. Armijos, Y. Fang, S. Garcia, K. Kelley, and S. Wright. 2009. Role of communally nesting ardeid birds in the epidemiology of West Nile virus revisited. Vector Borne Zoonotic Dis. 9: 275–280.

Richards, S. L., S. L. Anderson, and C. C. Lord. 2014. Vector competence of *Culex pipiens quinquefasciatus* (Diptera: Culicidae) for West Nile virus isolates from Florida. Trop. Med. Int. Health 19: 610–617.

Romo, H., J. L. Kenney, B. J. Blitvich, and A. C. Brault. 2018. Restriction of Zika virus infection and transmission in *Aedes aegypti* mediated by an insect-specific flavivirus. Emerg. Microbes Infect. 7: 181.

Smith, K. A., G. D. Campbell, D. L. Pearl, C. M. Jardine, F. Salgado-Bierman, and N. M. Nemeth. 2018. A retrospective summary of raptor mortality in Ontario, Canada (1991-2014), including the effects of West Nile Virus. J. Wildl. Dis. 54: 261–271.

Swetnam, D., S. G. Widen, T. G. Wood, M. Reyna, L. Wilkerson, M. Debboun, D. A. Symonds, D. G. Mead, B. J. Beaty, H. Guzman, et al. 2018. Terrestrial bird migration and West Nile Virus circulation, United States. Emerg. Infect. Dis. 24: 2184–2194.

Thiemann, T., B. Nelms, and W. K. Reisen. 2011. Bloodmeal host congregation and landscape structure impact the estimation of female mosquito (Diptera: Culicidae) abundance using dry ice-baited traps. J. Med. Entomol. 48: 513–517.

Thompson, J. N. 2005. Coevolution: the geographic mosaic of coevolutionary arms races. Curr. Biol. 15: R992–R994.

Weaver, S. C., T. W. Scott, and R. Rico-Hesse. 1991. Molecular evolution of eastern equine encephalomyelitis virus in North America. Virology. 182: 774–784.

Weingartl, H. M., J. L. Neufeld, J. Copps, and P. Marszal. 2004. Experimental West Nile virus infection in blue jays (*Cyanocitta cristata*) and crows (*Corvus brachyrhynchos*). Vet. Pathol. 41: 362–370.

Wheeler, S. S., C. M. Barker, Y. Fang, M. V. Armijos, B. D. Carroll, S. Husted, W. O. Johnson, and W. K. Reisen. 2009. Differential impact of West Nile Virus on California birds. Condor. 111: 1–20.

Wheeler, S. S., M. P. Vineyard, L. W. Woods, and W. K. Reisen. 2012a. Dynamics of West Nile virus persistence in House Sparrows (*Passer domesticus*). Plos Negl. Trop. Dis. 6: e1860.

Wheeler, S. S., S. A. Langevin, A. C. Brault, L. Woods, B. D. Carroll, and W. K. Reisen. 2012b. Detection of persistent west nile virus RNA in experimentally and naturally infected avian hosts. Am. J. Trop. Med. Hyg. 87: 559–564.

Worwa, G., A. A. Hutton, M. Frey, N. K. Duggal, A. C. Brault, and W. K. Reisen. 2018. Increases in the competitive fitness of West Nile virus isolates after introduction into California. Virology. 514: 170–181.

Worwa, G., A. A. Hutton, A. C. Brault, and W. K. Reisen. 2019. Comparative fitness of West Nile virus isolated during California epidemics. Plos Negl. Trop. Dis. 13: e0007135.

Zink, S. D., G. A. Van Slyke, M. J. Palumbo, L. D. Kramer, and A. T. Ciota. 2015. Exposure to West Nile Virus increases bacterial diversity and immune gene expression in *Culex pipiens*. Viruses. 7: 5619–5631.

Zou, G., B. Zhang, P. Y. Lim, Z. Yuan, K. A. Bernard, and P. Y. Shi. 2009a. Exclusion of West Nile virus superinfection through RNA replication. J. Virol. 83: 11765–11776.

Zou, G., F. Puig-Basagoiti, B. Zhang, M. Qing, L. Chen, K. W. Pankiewicz, K. Felczak, Z. Yuan, and P. Y. Shi. 2009b. A single-amino acid substitution in West Nile virus 2K peptide between NS4A and NS4B confers resistance to lycorine, a flavivirus inhibitor. Virology. 384: 242–252.